

## Assessing anti-predatory chemical defences among ten eastern Mediterranean sponges

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The palatability of organic chemical extracts from ten of the most abundant sponge species along the Israeli (shallow) coast, eastern Mediterranean Sea (*Axinella* sp., *Axinella polypoides*, *Chondrilla nucula*, *Ircinia* sp., *Psammocinia* sp. 1, *Psammocinia* sp. 2, *Psammocinia* sp. 3, *Psammocinia* sp. 4, *Sarcotragus* sp. and *Tetilla* sp.) was tested. To examine the generality of the phenomenon, it was evaluated with two types of potential predators, a fish and a gastropod. It was determined that the extracts of only two species (*Psammocinia* sp. 1 and *Psammocinia* sp. 3) deterred feeding of the omnivorous ornate Mediterranean wrasse *Thalassoma pavo*. On the other hand, extracts of five other sponges (*Chondrilla nucula*, *Axinella* sp., *Ircinia* sp., *Sarcotragus* sp. and *Psammocinia* sp. 2) were non-palatable to the omnivorous gastropod *Strombus persicus* (the extracts that deterred the fish did not deter the gastropod and vice versa). We also determined the capacity of extracts from six Red Sea sponges to deter *T. pavo*, and compared it with these extracts' effect on the Red Sea wrasse *T. klunzingeri*. All the extracts that deterred the Red Sea wrasse (from *Amphimedon chloros*, *Crella cyatophora*, *Negombata magnifica* and *Theonella swinhonis*) were also non-palatable to the Mediterranean wrasse. In addition to these four species, also *Diacarnus erythraenus* deterred *Thalassoma pavo* while being palatable to *T. klunzingeri*, whereas food pellets with extracts of *Niphates rowi* were eaten by both wrasse species.

### INTRODUCTION

Of all the marine invertebrates, sponges provide the most diverse and rich plethora of secondary metabolites (Faulkner, 2002; Paul et al., 2006). In recent years, studies have shown that secondary metabolites play an important role in an organism's ecology, including its survival (Ianora et al., 2006). Marine sponges have a high nutritional value, but most appear nonetheless to be minimally affected by predators (Kubanek et al., 2002). This has been suggested to be the result of their chemical defence mechanisms (Pawlik et al., 1995). Marine secondary metabolites may also act as antifouling agents (Kubanek et al., 2002; Bryan et al., 2003; Iken et al., 2003); in defence against bacterial pathogens (Kelman et al., 2001; Engel et al., 2002), fungal pathogens (Dube et al., 2002), viral attack (Mayer & Hamann, 2005), and fouling by diatoms (Amsler et al., 2000); in protecting from solar UV radiation (Stachowicz & Lindquist, 1997); in larval signalling (Zimmer & Butman, 2000; Stainberg & de Nys, 2002); and in allelopathic activity (Thacker et al., 1998; Kubanek et al., 2002; Pawlik et al., 2007) and more. The most commonly hypothesized and studied function, however, is the involvement of secondary metabolites in predator-prey interactions, especially in the benthic realm (Ianora et al., 2006). Organisms that are unable to move or hide, such as sponges, often exhibit predator avoidance techniques employing physical or chemical defences, while some are simply of low nutritional value which may enhance the effectiveness of their other defences (Duffy & Paul, 1992).

While past assessment of secondary metabolites activity was achieved by examining either antimicrobial activity (mainly inhibition of *Escherichia coli* growth) or ichthyotoxicity assays, during the last two decades studies have concentrated more on ecologically relevant assays (Hay, 1996; Paul et al., 2006). Palatability is defined as the acceptability of an item for ingestion. In palatability tests, contrasting results have been obtained depending on the potential predator used to evaluate anti-predatory effects of sponge chemical extracts (Pennings et al., 1994; Uriz et al., 1996; Waddell & Pawlik, 2000a,b). Therefore, it is necessary to seek a suitable and relevant test organism to understand the role and importance of defence mechanisms. It is accepted that generalist predators (preferably sympatric) are adequate as test organisms since mechanisms to circumvent defences would be less likely to evolve in a generalist than in a specialist predator (Pawlik et al., 1995). In addition, to enable comparison between different geographical regions it is advisable to use congeneric predator species (Burns et al., 2003).

In coral reefs the dominant potential predators of sponges are fish (Randall & Hartman, 1968; Wulff, 1994), while in Antarctic benthic communities sea-stars are the dominant spongivores (Furrow et al., 2003), and in temperate subtidal regions sea urchins are the dominant grazers. In south-eastern Australia for example, gut analysis has revealed that the sea urchin *Centrostephanus rogersii*, which can reach densities of up to 22 m<sup>-2</sup>, feeds on sponges in addition to consuming a broad range of algae (Wright et al., 1997).

Investigators have hypothesized that prey defences are better developed in tropical than in temperate habitats, because of increased predation pressure along the decreasing latitude; evidence for this phenomenon has been found in a wide range of terrestrial and marine systems (reviewed in Bolser & Hay, 1996). However, there is limited evidence that anti-predatory defence in sponges does indeed decrease closer to the poles (Hill et al., 2005). Recent studies have suggested that one or more evolutionary forces have acted in a species-specific manner irrespective of the geographical area (Becerro et al., 2003). In a study that compared anti-predatory chemical defence of sponges from the Mediterranean Sea and Guam, tropical and temperate sponges showed a comparable deterrence to global, sympatric and allopatric predators (Becerro et al., 2003).

The present study evaluated the anti-predatory chemical defence among sponges along the Israeli shore of the eastern Mediterranean Sea. We examined the chemical deterrence of ten abundant Mediterranean sponges against two omnivores, the ornate Mediterranean wrasse *Thalassoma pavo* and the gastropod *Strombus persicus*. We also examined whether intraspecific diversity in the deterrence of *T. pavo* exists within one chosen sponge species. Finally, the reaction of *T. pavo* to six Red Sea sponge extracts was compared with the reaction of *T. klunzingeri* to extracts from the same sponges.

## MATERIALS AND METHODS

### *Sponge collection and identification*

Sponges were collected by SCUBA diving in Sdot-Yam, eastern Mediterranean Sea, Israel ( $32^{\circ}29.77'N$   $34^{\circ}53.23'E$ ), between March and August. Parts of the sponges (upper 75%) were dissected by using a surgical blade and placed in zip-lock bags, leaving the rest of the sponge attached to the substrate, allowing recuperation and regeneration. Only sponges larger than 150 ml were sampled (*Chondrilla nucula*, *Axinella polypoides*, *Axinella* sp. and *Teilla* sp. N=4, *Psammocinia* sp. 3 N=5, and for the rest N=1). The samples were stored in a cooler and then frozen at  $-20^{\circ}C$  for later use. A sub-sample from each specimen was fixed and preserved for morphological identification based on the appropriate literature (Hooper & van Soest, 2002).

### *Tissue extraction*

Frozen sponge samples were cut into approximately 1 cm<sup>3</sup> cubes. The sample volume was measured by water displacement. Samples were put in a 1:1 mixture of dichloromethane (DCM) and methanol, kept at 40°C for 24 h and were shaken periodically. The methanol dissolved the cell membranes and allowed their content to mix with the DCM. After extraction, both (methanol/water and DCM) phases were passed through filter papers (Whatman), evaporated to dryness on a rotary evaporator at low heat (<50°C), and then combined. The process was repeated twice. A third extraction with methanol was carried out for 6 h at room temperature. The resulting extract was filtered and evaporated in the same manner. Since the food pellets in the feeding assays were designed to closely mimic the secondary metabo-

lites content within a sponge, crude extracts from all three stages were combined and stored at  $-20^{\circ}C$  until further use. The natural concentration of the metabolites extracted from the sponge was calculated by dividing the dry (lyophilized) weight of the crude extract by the volume of the extracted tissue.

### *Fish feeding experiment*

#### *Food preparation*

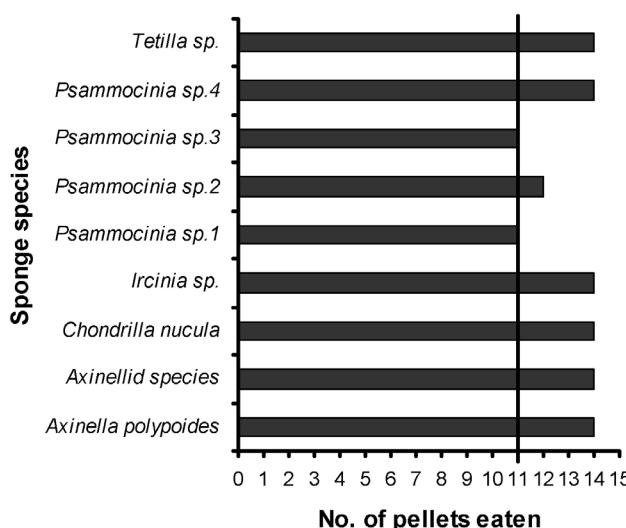
Aquarium assays were conducted following Burns et al. (2003) with minor modifications.

To obtain food pellets for the experiments, crude organic extract from 7 ml of each sponge species was dissolved in DMSO added to a mixture of 0.3 g alginic acid (Sigma), 0.5 g of freeze-dried powdered squid mantle and distilled water to yield a final volume of 7 ml (Pawlak et al., 1995). The mixture was stirred until the extract was homogeneously distributed in the alginic acid, and then loaded into a syringe. The content of the syringe was emptied into a 0.25 M calcium chloride solution, forming a long spaghetti-like strand. After the strand hardened it was removed from the solution, rinsed in seawater and cut into 3–4 mm long pellets using a razor blade. Control pellets were made identically, but without addition of the extracts. It was found during preliminary experiments that pellet colour did not affect food acceptability by *Thalassoma pavo* and therefore food colouring was not used.

#### *Feeding assays*

Control and treatment pellets were presented to pairs of Mediterranean wrasses *Thalassoma pavo*. The ornate wrasse *T. pavo* is a labrid fish inhabiting shallow rocky bottoms in the Mediterranean Sea (Guidetti et al., 2002). It is a generalist carnivore with a tendency to omnivory (Kabasakal, 2001). The merits for using a generalist fish are described in Pawlik et al. (1995). Moreover, fish from the same genus have been used in similar studies (e.g. *T. bifasciatum*, Pawlik & Fenical, 1992; *T. klunzingeri*, Burns & Ilan, 2003, Burns et al., 2003; and *T. pavo*, Marin et al., 1998, Tsoukatou et al., 2002).

The fish were held in separate opaque-sided compartments in a running seawater system provided with aeration. Several rocks were introduced to create refuges in order to simulate a more natural environment. The fish were considered acclimatized to the experimental set-up when they freely approached and ate control food pellets. Fish were then presented with another control pellet. If the pellet was consumed, a treated food pellet was then presented. If the treated pellet was rejected, an additional control pellet was offered, to ascertain that the fish was still hungry (results of satiated fish were not analysed). A pellet was considered rejected if it was not eaten after a minimum of three attempts by one or more fish, or if it was approached and ignored after a single attempt. The significance of differences in the consumption of treated versus control pellets was evaluated using Fisher's exact test. Experiments were conducted with ten or 15 replicates (Red Sea and Mediterranean sponges respectively). A treatment was considered significantly deterrent if four or more pellets out of ten or 15 were rejected ( $P<0.0433$ , and  $P<0.0498$ , 1-tailed test, respectively).



**Figure 1.** Palatability of eastern Mediterranean sponge chemical extracts to the Mediterranean wrasse *Thalassoma pavo*. Significant deterrence is indicated when the histogram bars fall to the left of the perpendicular line on the figure ( $P<0.0498$ , Fisher's exact test, 1-tailed).

#### Gastropod feeding experiment

##### Food preparation

Assays using the gastropod *Strombus persicus* were based, with modifications, on a method described earlier (Hay et al., 1994). *Strombus persicus* is an exotic species new to the eastern Mediterranean Sea (Gofas & Zenetos, 2003). *Strombus persicus* inhabits a low-energy environment of shallow bays, on mixed (rock/sand/mud) bottom (Zenitos et al., 2003). While moving over the substrate it collects soft organic matter and detritus from the bottom surface by movements of its large flexible proboscis (Alyakrinskaya, 2003). The gastric contents of other *Strombus* species have included spicules of sponges, radiolarians, diatoms and very small crustaceans (Alyakrinskaya, 2003). The gastropods were collected from shallow waters (3–4 m depth), at Michmoret, eastern Mediterranean Sea, Israel. They were divided randomly into ten separate aquaria, five individuals in each tank, with artificial seawater and aeration. Water was changed once a week or after concluding an experiment.

Artificial food was prepared by boiling 0.2 g granulated agar with 20 ml distilled water; when the liquid temperature dropped to 40°C, 1.5 g of freeze-dried powdered squid mantle was added. Treatment food included crude organic extract from 20 ml of each sponge species. The extracts were dissolved in 1.5 ml ethanol and 1.5 ml filtered seawater. Extract was added immediately after mixing the powdered squid mantle into the liquid agar after it cooled, but before it solidified. In this case agar rather than alginic acid was used following Hay et al. (1994) who found it affective for this type of strips preparation (see below). Control food was prepared similarly (including the solvents) but was devoid of the chemical extract. The mixture was stirred until the extract was homogeneously distributed in the agar and poured into a rectangular wooden mould (24.5×3.5 cm with a divider in the middle) attached to a glass surface, for coagulation. A section of window screen was clamped between the mould and the glass surface to form a base for the artificial

food. Control food was poured into one half of the frame and treatment food was poured into the other half. The cooling agar took the shape of the mould and became firmly attached to the screen. The screen was then gently removed from the mould and cut into 1 cm wide strips to obtain 12 strips of treatment and 12 of control food. The food strips were numbered and weighed. Two small rust-proof bolts were tied to each food strip to prevent buoyancy.

##### Feeding assays

During an experiment, each aquarium contained one control food strip and one treatment food strip. These were left in the aquaria for a period of 18–19 hours, after which they were removed, dried lightly with a paper towel and weighed without the bolts. To control for autogenic changes, each experiment included a control aquarium with two strips of each kind of food type (control and treatment) and no gastropods. This was done in order to allow measurement of any weight change in the food strips that might have taken place during the experimental period, that was not a consequence of the gastropods' consumption.

The percentage of food consumed by *S. persicus* of each food strip was calculated based on weight loss. The percentage lost from food strips with no gastropods was subtracted from the percentage lost in aquaria with gastropods, for both control and treatment food strips. Results were analysed using a Wilcoxon paired-sample test (Zar, 1999).

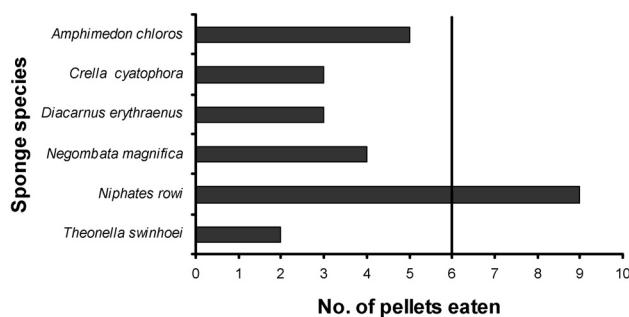
## RESULTS

### Fish experiments

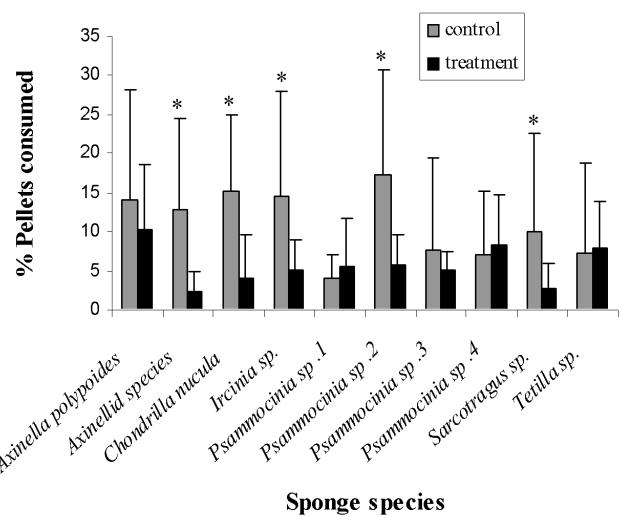
Laboratory bioassays with the fish *Thalassoma pavo* were conducted with ten abundant sponge species from the Israeli coast of the Mediterranean Sea (identified as: *Axinella* sp., *Axinella polypoides*, *Chondrilla nucula*, *Ircinia* sp., *Psammocinia* sp. 1, *Psammocinia* sp. 2, *Psammocinia* sp. 3, *Psammocinia* sp. 4, *Sarcotragus* sp. and *Tetilla* sp.). Bioassays revealed the existence of non-palatable secondary metabolites in the extracts of only two of these sponge species: *Psammocinia* sp. 3 and *Psammocinia* sp. 1 (Figure 1), which were consumed significantly less than the control food pellets ( $P<0.05$ ).

*Sarcotragus* sp. had been chosen a priori to assess the existence of intraspecific variation in its ability to deter *Thalassoma pavo*, because of its high abundance in the research area, which allowed the collection of several large specimens. However, the five examined species did not deter predation by *T. pavo* ( $P>>0.05$ ).

Since only two of the Mediterranean species had been found to be marginally deterrent to fish, it was decided to re-evaluate the suitability of using *T. pavo* as a potential predator in these experiments. Six Red Sea sponge species, whose deterrent capability had been previously determined by using *Thalassoma kyunzingeri* in a similar protocol (Burns et al., 2003), were therefore extracted and tested with *T. pavo*. Four species whose extracts deterred predation by *T. kyunzingeri* were selected: *Amphimedon chloros*, *Crella* (*Grayella*) *cyatophora*, *Negombata magnifica* and *Theonella swinhonis*. Two additional species (*Diacarnus erythraenus* and *Niphates rowi*) were previously found to be non-deterrent against *Thalassoma kyunzingeri* (Burns et al., 2003). Upon assessing deterrence activity of these six Red Sea sponges against *T. pavo*, five



**Figure 2.** Palatability of Red Sea sponge chemical extracts to the Mediterranean wrasse *Thalassoma pavo*. Significant deterrence is indicated by the perpendicular line on the figure ( $P<0.043$ , Fisher's exact test, 1-tailed).



**Figure 3.** Palatability of eastern Mediterranean sponge chemical extracts to the gastropod *Strombus persicus*. \*, Significant deterrence (Wilcoxon's paired-sample test,  $P<0.05$ ).

species were deterrent, as pellets with their chemical extracts were consumed significantly less ( $P<0.05$ ), while only the extract of *Niphates rowi* did not deter predation by this fish (Figure 2).

#### Gastropod experiments

Chemical extracts of ten sponge species were examined to determine their capability to deter predation by the gastropod *Strombus persicus*. Food strips with extracts from five species (*Chondrilla nucula*, *Axinella* sp., *Ircinia* sp., *Sarcotragus* sp., *Psammocinia* sp. 2) were grazed significantly less ( $P<0.05$ ) by *Strombus persicus* than control food strips, indicating that these sponge species deterred predation by this gastropod while the rest were chemically non-deterrent (Figure 3).

#### DISCUSSION

The present study examined anti-predatory chemical defence in ten sponges from the Israeli coast along the eastern Mediterranean Sea. Only two (20%) of these sponges (*Psammocinia* sp. 1 and *Psammocinia* sp. 3) deterred the generalist predator, the ornate wrasse *Thalassoma pavo* (Figure 1). This is much lower than the 69% found in the

Bahamas (Pawlik et al., 1995) or 41% among Red Sea sponges (Burns et al., 2003). In both latter cases, the predators used were of the genus *Thalassoma*. Similarly, the predator in the current study was of the same genus, *T. pavo*. The fact that extracts of Red Sea sponges deterred *T. pavo* supports the findings with the Mediterranean extracts, and reinforces the use of this fish as appropriate in such experiments. The different reactions of *T. pavo* and *T. klunzingeri* to the extract of *Diacarnus erythraeus* have several possible explanations. Active secondary metabolites might vary between seasons (Becerro et al., 1997), and intraspecific diversity might also account for the different results of the two predators (Hay, 1996).

Becerro et al. (2003) compared chemical anti-predatory defence in sponges from the (temperate) western Mediterranean Sea and from (tropical) Guam, and found no relation between chemical defence and geographical location. Their results could not support the hypothesis that there is a latitudinal gradient in the production of chemical defences as a response to increased predation pressure closer to the tropics. The eastern Mediterranean sponges, however, indeed exhibit less anti-predatory chemical defence than the western Mediterranean species. Although the east Mediterranean has lower species diversity than the west, the relative lack of anti-fish chemical defence in sponges in the current study cannot be explained solely by absence of predation pressure.

The fact that *T. pavo* was not deterred by natural products from eight of the species could be explained by the existence of alternative anti-predatory defence mechanisms. For example, species from the Irciniidae (*Ircinia* sp., *Psammocinia* sp. 2, *Psammocinia* sp. 4, *Sarcotragus* sp.) bear fine collagenous (spongillin) filaments that supplement their fibre skeleton and (the latter genus) may have surface covered with sand (Cook & Bergquist, 2002). When present in sufficient quantities, these filaments make the sponges very difficult to tear (Cook & Bergquist, 2002) and they may also reduce their nutritional quality as prey, since they are hard to digest (Duffy & Paul, 1992; Chanas & Pawlik, 1996). This kind of anti-predatory defence could be sufficient to protect sponges from fish such as *T. pavo*.

Various species from the genus *Ircinia* showed different degrees of palatability (Van der Vyver et al., 1990; Pawlik et al., 1995; Epifanio et al., 1999; Tsoukatou et al., 2002; Becerro et al., 2003), indicating the existence of different anti-predatory mechanisms. The species *Axinella* sp. and *Tetilla* sp. both have a thin external sediment 'armour' that can prevent fish from feeding on them. As for the *Tetilla* sp. and *Axinella polypoides*, the large spicules they contain (van Soest & Hooper, 2002a,b) could also serve as an anti-predatory mechanism. A study conducted in the Red Sea showed that spicules exceeding  $\sim 250 \mu\text{m}$  deterred predation by *Thalassoma klunzingeri* (Burns & Ilan, 2003). While chemical extract of *Chondrilla nucula* in the present study was palatable to the wrasse, extract of *C. nucula* from the Bahamas, deterred predation by *T. bifasciatum* (Pawlik et al., 1995). This could be explained by geographical variability in chemical content. In addition, *C. nucula* could have alternative protection since it has a tough exterior that would be difficult for fish to tear.

Different consumers differ in their sensitivity to secondary metabolites (Pennings et al., 1994; Waddell & Pawlik, 2000a,b). Predator behaviour and preference may also determine the outcome of feeding experiments, clearly affecting how we perceive the deterrent properties of our samples (Becerro et al., 2003). The activity of the sponge extracts assayed with the ornate wrasse *T. pavo* was also examined against the gastropod *Strombus persicus*. In these assays more extracts deterred the gastropod than those that deterred the fish. It is interesting to note that the two sponge extracts that deterred *T. pavo* did not deter *S. persicus* (and vice versa). In a study that assessed the ability of crude sponge extracts to deter fish (*T. lunzingeri*) and sea urchin (*Diadema setosum*), all the extracts that inhibited fish predation were also non-palatable to the sea urchin, whereas some extracts only deterred the sea urchin (Burns et al., 2003). In the current study, on the other hand, it is interesting to note that the sponges that deterred the fish were palatable to the gastropod, and those that were rejected by the gastropod were eaten by the fish. Since gastropods graze and fish bite, it is reasonable to assume that they react to different anti-predatory mechanisms. The deterrence pattern in the present study indicates that the compound active against each of these potential predators is different, and that the sponges can maintain a diverse arsenal of compounds to protect them from various predators.

Studies dealing with anti-predatory chemical defence have given different results according to the predator chosen to assess the anti-predatory activity (Pennings et al., 1994; Bryan et al., 1997; McClintock & Baker, 1997; Waddell & Pawlik, 2000a,b). For this reason it is necessary to choose a suitable test organism that will be a relevant potential predator (Uriz et al., 1996). The main groups of predators usually used to examine deterrence activity of sponge extracts are fish (e.g. Pennings et al., 1994; Wulff, 1994; Pawlik et al., 1995; Marin et al., 1998; Epifanio et al., 1999; Tsoukatou et al., 2002; Kubanek et al., 2002; Becerro et al., 2003; Burns et al., 2003), while fewer studies have concentrated on invertebrates such as echinoderms, mainly sea-stars (Waddell & Pawlik, 2000b; Amsler et al., 2001; Furrow et al., 2003), urchins (Uriz et al., 1996; Becerro et al., 1997; Wright et al., 1997; Burns et al., 2003), and crabs (Pennings et al., 1994; Waddell & Pawlik, 2000a), and nudibranchs, although the latter are specialist predators (Becerro et al., 1998; Gemballa & Schermutzki, 2004). Several studies indicated that the use of more than one group of predators can produce contrasting results, reflecting that a diversity of defensive secondary metabolites have evolved in sponges.

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